transfected transgene in both RNA and protein products. In this fashion, other pairs of drug selection expression vectors may be employed to produce cell lines transfected with multiple vectors which have stably integrated high copy numbers of the genetic material and express various levels of biologically active molecules of interest.

A multiplicity of cells may be used in the methods of this invention, such that implantation of a polymer-capsule can be sufficient to provide an effective amount of the needed substance or function to an individual. In addition, more than one biologically active molecule may be stably expressed and/or delivered over long periods from a single capsule.

One way to accomplish this result is to encapsulate a single cell line which has been genetically altered to express more than one heterologous gene.

Another way to accomplish this result is to encapsulate in a single capsule a mixture of cells, wherein some cells have been genetically modified to express one biologically active molecule and other cells have been genetically modified to express a second biologically active molecule. It will be appreciated that a non-genetically engineered cell line can be utilized to provide the second biologically active molecule.

For example, a cell line may also be genetically engineered to express different biologically active molecules. The sub clones of the parental cell lines, each expressing a different transgene, may then be pooled and encapsulated to achieve the desired effect on a long-term basis.

These approaches would eliminate the need for using multiple implants for the long-term, stable

expression and delivery of more than one biologically active molecule to a single site.

This invention also contemplates using capsules containing cells that are genetically modified with a heterologous gene, which gene enables the cells to remain viable within the capsule upon implantation within a host mammal. In other words, the methods of this invention are also directed to methods of delivery of molecules within the implanted capsules.

In a specific embodiment of this invention we used the pNUT expression vector containing the human ß-NGF gene operatively linked to the mouse metallothionein promoter to transfect BHK cells via the calcium phosphate co-precipitation method.

A variety of biocompatible immunoisolatory capsules are suitable for delivery of molecules according to this invention. Such capsules will allow for the passage of metabolites, nutrients and therapeutic substances while minimizing the detrimental effects of the host immune system. Preferably the capsule of this invention will be similar to those described in Aebischer et al., PCT publication WO 92/19195, incorporated herein by reference.

Most preferably the T1/2 membranes described herein are used to encapsulate the cells that are modified according to the methods of this invention. It will be appreciated that the T1/2 membranes described herein can also be used for encapsulation of any other suitable cell or cell lines. Thus, the T1/2 membranes of this invention can be used to encapsulate primary (non-dividing) cells, as well as dividing cells.

Useful biocompatible polymer capsules comprise (a) a core which contains a cell or cells, either suspended in a liquid medium or immobilized

within a hydrogel or extracellular matrix, and (b) a surrounding or peripheral region of permselective matrix or membrane (jacket) which does not contain isolated cells, which is biocompatible, and which is sufficient to protect isolated cells if present in the core from detrimental immunological attack.

The core of the polymer capsule is constructed to provide a suitable local environment for the continued viability and function of the cells isolated therein.

Many transformed cells or cell lines are most advantageously isolated within a capsule having a liquid core. For example, cells can be isolated within a capsule whose core comprises a nutrient medium, optionally containing a liquid source of additional factors to sustain cell viability and function, such as fetal bovine or equine serum.

Microcapsules may sometimes be suitable for use in the methods and compositions of this invention. The fabrication of microcapsules have been described in Espevik et al., PCT publication WO 9107951, and Sefton, United States patent 4,353,888 incorporated herein by reference.

Suitably, the core may be composed of a matrix formed by a hydrogel which stabilizes the position of the cells in cell clumps. The term "hydrogel" herein refers to a three dimensional network of cross-linked hydrophilic polymers. The network is in the form of a gel, substantially composed of water, preferably but not limited to gels being greater than 90% water.

Compositions which form hydrogels fall into three classes. The first class carries a net negative charge (e.g., alginate). The second class carries a net positive charge (e.g., collagen and laminin).

Examples of commercially available extracellular matrix components include Matrigel™ and Vitrogen™. Fibroblasts generally survive well in a positively charged matrix and are thus suitably enclosed in extracellular-matrix type hydrogels. The third class is net neutral in charge (e.g., highly crosslinked polyethylene oxide, or polyvinylalcohol). Any suitable matrix or spacer may be employed within the core, including precipitated chitosan, synthetic polymers and polymer blends, microcarriers and the like, depending upon the growth characteristics of the cells to be encapsulated.

Preferably, the capsules are immunoisolatory. To be immunoisolatory, the surrounding or peripheral region of the capsule should confer protection of the cells from the immune system of the host in whom the capsule is implanted, by preventing harmful substances of the host's body from entering the core of the vehicle, and by providing a physical barrier sufficient to prevent detrimental immunological contact between the isolated cells and the host's immune system. The thickness of this physical barrier can vary, but it will always be sufficiently thick to prevent direct contact between the cells and/or substances on either side of the barrier. The thickness of this region generally ranges between 5 and 200 microns; thicknesses of 10 to 100 microns are preferred, and thickness of 20 to 75 microns are particularly preferred. Types of immunological attack which can be prevented or minimized by the use of the instant vehicle include attack by macrophages, neutrophils, cellular immune responses (e.g. natural killer cells and antibody-dependent T cell-mediated cytoloysis (ADCC), and humoral response (e.g., antibody-dependent complement mediated cytolysis).

Use of immunoisolatory capsules allows the implantation of xenogeneic cells or tissue, without a concomitant need to immunosuppress the recipient. Use of immunoisolatory capsules also allows use of unmatched cells (allografts). The type and vigor of an immune response to xenogeneic cells is expected to differ from the response encountered when syngeneic or allogeneic tissue is implanted into a recipient. This response may proceed primarily by cell-mediated, or by complement-mediated attack; the determining parameters in a particular case may be poorly understood. However, the exclusion of IgG from the core of the vehicle is not the touchstone of immunoprotection, because in most cases IgG alone is insufficient to produce cytolysis of the target cells or tissues. Using immunoisolatory macrocapsules, it is possible to deliver needed high molecular weight products or to provide metabolic functions pertaining to high molecular weight substances, provided that critical substances necessary to the mediation of immunological attack are excluded from the immunoisolatory capsule. These substances may comprise the complement attack complex component Clq, or they may comprise phagocytic or cytotoxic cells; the instant immunoisolatory capsule provides a protective barrier between these harmful substances and the isolated cells. Thus, an immunoisolatory capsule can be used for the delivery even from xenogeneic cells, products having a wide range of molecular sizes, such as insulin, parathyroid hormone, interleukin 3, erythropoietin, albumin, transferrin, enkephalins, endorphins, catecholamines, Factor VIII, NGF, BDNF, NT-3, NT-4/5, CNTF, GDNF, CDF/LIF, EGF, IGF, bFGF, aFGF, PDGF, TGF and the like.

Various polymers and polymer blends can be used to manufacture the capsule jacket, including

polyacrylates (including acrylic copolymers), polyvinylidenes, polyvinyl chloride copolymers, polyurethanes, polystyrenes, polyamides, cellulose acetates, cellulose nitrates, polysulfones, polyphosphazenes, polyacrylonitriles, poly(acrylonitrile/covinyl chloride), as well as derivatives, copolymers and mixtures thereof.

The capsule can be any configuration appropriate for maintaining biological activity and providing access for delivery of the product or function, including for example, cylindrical, rectangular, disk-shaped, patch-shaped, ovoid, stellate, or spherical. Moreover, the capsule can be coiled or wrapped into a mesh-like or nested structure. If the capsule is to be retrieved after it is implanted, configurations which tend to lead to migration of the capsules from the site of implantation, such as spherical capsules small enough to travel in the recipient's blood vessels, are not preferred. Certain shapes, such as rectangles, patches, disks, cylinders, and flat sheets offer greater structural integrity and are preferable where retrieval is desired.

The instant capsule must provide, in at least one dimension, sufficiently close proximity of any isolated cells in the core to the surrounding tissues of the recipient, including the recipient's bloodstream, in order to maintain the viability and function of the isolated cells. However, the diffusional limitations of the materials used to form the capsule do not in all cases solely prescribe its configurational limits. Certain additives can be used which alter or enhance the diffusional properties, or nutrient or oxygen transport properties, of the basic vehicle. For example, the internal medium can be

supplemented with oxygen-saturated perfluorocarbons, thus reducing the needs for immediate contact with blood-borne oxygen. This will allow isolated cells to remain viable while, for instance, a gradient of angiotensin is released from the capsule into the surrounding tissues, stimulating ingrowth of capillaries. References and methods for use of perfluorocarbons are given by Faithful, N.S. (1987) Anaesthesia, 42, pp. 234-242 and NASA Tech Briefs MSC-21480, U.S. Govt. Printing Office, Washington, D.C. 20402, incorporated herein by reference.

In one preferred embodiment, the implantable capsule is of a sufficient size and durability for complete retrieval after implantation. Such macrocapsules have a core of a preferable minimum volume of about 1 to 10μl and depending upon use are easily fabricated to have a volume in excess of 100 μl.

The preferred capsule will have an inner single ultrafiltration membrane with a permselective pore-size permeability range of 60-98% BSA rejection coefficient and 50-90% ovalbumin rejection coefficient. The capsule may be in the form of a flat sheet sealed at the periphery or of a hollow fiber sealed at the ends as described in PCT application WO 92/19195. In a flat sheet format the two walls will be separated by a gap thickness of less than 1000 microns, preferably less than 300 microns. Wall thickness should be between about 25-200 microns, preferably between about 30-75 microns.

In a hollow fiber configuration, the fiber will have an inside diameter of less than 1500 microns, preferably less than 300-600 microns. In either geometry, the hydraulic permeability will be in the range of 1-100 mls/min/M^2/mmHg, preferably in the range of 25 to 70 mls/min/M^2/mmHg. The glucose mass transfer

coefficient of the capsule, defined, measured and calculated as described by Dionne et al., ASAIO Abstracts, p. 99 (1993), and Colton et al., The Kidney, eds., Brenner BM and Rector FC, pp. 2425-89 (1981) will be greater than 10^{-6} cm/sec, preferably greater than 10^{-4} cm/sec.

The morphology of the outer wall surface of the capsule is variable. Previously described T1, T2, T4 membranes and the novel T1/2 membranes of this invention differ in their outer wall surface morphology. All these membranes are characterized by an inner permselective skin.

T1 membranes are characterized by an "open" or fenestrated non-permselective outer surface wall, and a trabecular wall structure between the outer and inner wall surfaces. See, e.g. Lacy et al., Science, 254, pp. 1782-84 (1991). The fenestrations of "macropores" in the outer wall surface of a T1 membrane typically occupy about 20%-40% of the total outer surface wall area. Typically, the macropores are 10μm-15μm in diameter or greater.

T2 membranes have a similar trabecular structure between the inner and outer walls but are characterized by a more "closed" or smoother outer surface wall. T2 membranes, typically are characterized by fewer than 10% macropores on the outer surface wall and virtually no macropores in the 5-15 μm diameter size range.

T4 membranes are further distinct in that the outer surface is also permselective, unlike the T1 or T2 membranes. T4 membranes are useful for CSF implantation sites, such as the ventricles or sub-arachnoid space, as well as other fluid bathed implantation sites.

For primate brain parenchyma, or other "solid" tissue implantation sites, we prefer to employ a smooth wall capsule which is substantially impermeable to cells. Others have described open walls which encourage microvascularization (see, e.g., Brauker et al., WO/92/07525). T1 and T1/2 capsules are especially suited for long term implants. We prefer T1/2 capsules.

T1/2 capsules are characterized by a total macropore distribution of between approximately 2-20%, preferably 2-15% of the total outer surface wall area. The macropores should fall within the size range of approximately 5 μm to about 15 μm in diameter. The relative distribution of pore sizes within this range can vary.

In one specific embodiment T1/2 hollow fiber membrane capsules made from PAN/PVC were utilized. According to this embodiment, the total macropore area was about 12% of the total outer wall surface area. Approximately 20% of the macropores ranged between 5-10 μm in diameter and about 80% of the macropores were about 10 μm in diameter.

In another specific embodiment, T1/2 hollow fiber membrane capsules were fabricated having a total macropore area of about 2.4% of the total outer wall surface area. Approximately 17% of the macropores were about 5 μm in diameter, about 33% were about 10 μm in diameter, and about 50% were about 15 μm in diameter.

In another specific embodiment, T1/2 hollow fiber membrane capsules were fabricated having a total macropore area of approximately 10% of the total outer wall surface area. Greater than 99% of these macropores ranged between 10-15 μm in diameter.

flow rates using well known techniques described by Cabasso, I., Encyclopedia of Chemical Technology, 12, pp. 492-517 (1980). The coagulant which can include biological tissue fragments, organelles, or suspensions of cells and/or other therapeutic agents, as described in Dionne, WO 92/19195 and United States Patents 5,158,881, 5,283,187 and 5,284,761, incorporated herein by reference.

According to those methods, T1 membranes may be formed by coextrusion of a polymer solution and coagulant solution through air before entering a quench bath. T2 membranes may be formed by coextruding the polymer, and coagulation solutions into humidified air or a mist and then into a bath. T4 membranes may be formed by coextrusion of the polymer and coagulant solutions directly into a coagulant bath, so that formation of the permselective membrane occurs on both outer and inner wall surfaces simultaneously.

T1/2 membranes may be formed using similar methods used to form T2 membranes. However, the mist or humidity at the coextrusion port may be controlled according to known methods to produced the desired outer surface morphology. Alternatively, the nozzle distance from a quench bath may be varied, according to routine methods. Further, if coextrusion is used to cast the membrane, the absolute and/or relative flow rates of polymer and coagulant may be adjusted to achieve the desired outer wall surface morphology. Finally, the polymer and coagulant solution compositions and temperatures can be varied to achieve the desired outer surface wall morphology. For example, the casting solution may be 10-15% PAN/PVC in DMSO (w/w) and the coagulant may be water, or other aqueous medium. Alternatively, the casting solution

may be, e.g., 16% PAN/PVC, and the coagulant may be, e.g, 40% NMP, 60% H_2O at 23°C.

Any suitable method of sealing the capsules may be used, including the employment of polymer adhesives and/or crimping, knotting and heat sealing. These sealing techniques are known in the art. In addition, any suitable "dry" sealing method can also be used. In such methods, a substantially non-porous fitting is provided through which the cell-containing solution is introduced. Subsequent to filling, the capsule is sealed. Such a method is described in copending United States application Serial No. 08/082,407, herein incorporated by reference.

The methods and devices of this invention are intended for use in a mammalian host, recipient, subject or individual, preferably a primate, most preferably a human.

A number of different implantation sites are contemplated for the devices and methods of this invention. These implantation sites include the central nervous system, including the brain, spinal cord, and aqueous and vitreous humors of the eye. Preferred sites in the brain include the striatum, the cerebral cortex, subthalamic nuclei and nucleus Basalis of Maynert. Other preferred sites are the cerebrospinal fluid, most preferably the subarachnoid space and the lateral ventricles. This invention also contemplates implantation into the kidney subcapsular site, and intraperitoneal and subcutaneous sites, or any other therapeutically beneficial site.

In an embodiment of this invention, methods are provided for the treatment of diseases caused by neural degeneration. Examples of human diseases which are thought to be associated with neural degeneration include Alzheimer's disease, Huntington's disease,

AIDS-related dementia, Amyotrophic Lateral Sclerosis (ALS) and Parkinson's disease.

Some animal models for neurodegenerative conditions are based on the premise that a specific insult may damage or kill neurons. In some cases this may even lead to a cascade of neuronal death which affects trophically interdependent neurons along pathways responsible for specific brain functions.

A strategy for treatment of neural degenerative conditions involves the localized administration of growth or trophic factors in order to (1) inhibit further damage to postsynaptic neurons, and (2) improve viability of cells subjected to the insult. Factors known to improve neuronal viability include NGF, BDNF, NT-3, NT-4/5, CNTF, GDNF, CDF/LIF, bFGF, aFGF, IGF, neurotensin, and Substance-P.

In one animal model for neurodegenerative excitotoxicity, the glutamate analog, quinolinic acid, is injected stereotaxically into the brain region known as the striatum and/or basal ganglia to produce neuropathology and symptoms analogous to those of patients suffering from Huntington's disease. Both the model and actual Huntington's disease are characterized by damage to neurons necessary for aspects of motor control. Furthermore, one of the early symptoms of Huntington's disease is loss of body weight (Sanberg, et al. Med J Aust., 1, pp. 407-409 (1981)). Similar effects are also seen in the model system (Sanberg, et al. Exp Neurol, 66, pp. 444-466 (1979)). Quinolinic acid is also found at abnormally high levels in humans suffering from AIDS-related dementia.

Huntington's disease (HD) is an autosomal dominant disorder characterized by a progressive dementia coupled with bizarre uncontrollable movements and abnormal postures. HD is found in nearly all

ethnic and racial groups with the prevalence rate in the U.S. approximately 50/1,000,000 (Emerich, D.F. & Sanberg, Neuromethods, 21, pp. 65-134 (1992)). The manifestation of the disorder typically occurs in middle life, about 35-45 years of age, followed by an intractable course of mental deterioration and progressive motor abnormalities with death usually occurring within 15 years. Research into the neural pathology in HD has revealed a complex mosaic of related and interdependent neurochemical and histopathological alterations.

A variety of avenues have been explored to develop an animal model of HD. Recent investigations have centered on the relationship between striatal damage and the locomotor abnormalities resulting from the use of selective cytotoxic compounds. Glutamate is one of the major excitatory neurotransmitters found in the CNS. It can act, however, as a potent neurotoxin and a number of attempts have been made to develop animal models of HD based on the relatively specific cytotoxic effects of glutamate and other excitotoxic compounds. These compounds include structural analogs of glutamate, such as kainic acid (KA), ibotenic acid (IA), and the endogenous tryptophan metabolite quinolinic acid (QA). When injected into the brains of rats, in extremely small doses, these compounds produce a marked and locally restricted toxic effect while sparing axons of passage and afferent nerve terminals. The behavioral, neurochemical, and anatomical consequences of excitotoxicity resemble those observed in HD and have led to the speculation that an aberrant overproduction or breakdown of endogenous excitotoxic compounds is an etiological factor in HD.

Quinolinic acid, 2,3-pyridine dicarboxylic acid, a metabolite of tryptophan, has attracted a great

deal of attention because of its powerful excitotoxic properties and wide distribution in both rat and human brain (Schwarcz and Kohler, Neurosci. Lett., 38, pp. 85-90 (1983); and Schwarcz et al., Science, 219, pp. 316-318 (1983)). High concentrations of its catabolic enzyme, quinolinic acid phosphoribosyltransferase (QPRT), and immediate anabolic enzyme, 3-hydroxyanthranilic acid (3HAO), have been detected within the caudate suggesting that it normally serves a role in striatal functioning (Foster et al., Brain Res., 336, pp. 207-214 (1985)). The striatum is among the structures most vulnerable to the excitotoxic effects of QA (Schwarcz and Kohler, (1983), supra) and neonatal, but not mature, animals appear to be resistant to the toxic effects of QA corresponding roughly to the typical onset of HD in middle age.

Quinolinic acid has been reported to exert a more selective degenerative effect in the striatum than KA, which more closely resembles the pathology of HD (Beal, Synapse, 3, pp. 38-47 (1989); and Roberts and DiFiglia, Synapse, 3, pp. 363-371 (1989)). Like KA, QA injections cause depletions of GABAergic neurons while relatively sparing cholinergic neurons and axons of extrinsic origin. Unlike KA or IA, intrastriatal injections of QA appear to spare somatostatin- and neuropeptide Y-containing neurons suggesting that this model most closely reproduces the neuropathology observed in the disease (Beal et al., J. Neuro Sci, 8, pp. 3901-3908 (1988).

According to the present invention, trophic factors are provided to the proper brain region by implanting a capsule containing cells, including genetically altered cells which secrete an appropriate factor. In some instances, the genetically altered

cells are autologous to the host and may not require encapsulation.

Nerve growth factor-secreting cells such as BHK cells engineered to express human NGF represent a therapy for quinolinic acid induced neurodegeneration.

Another animal model involves lesion of the fimbria-fornix (rodents) or fornix (primates). In particular, neurons of the septohippocampal system are axotomized which leads to NGF-dependent degeneration and cell death in the septal cholinergic neurons. These lesions cause degenerative changes in brain areas similarly affected in Alzheimer's disease in humans.

According to the methods of this invention, NGF may be delivered to the affected area by the implantation of a capsule containing genetically altered cells which secrete NGF. Other neurotrophic factors such as CNTF, BDNF, bFGF, CDF/LIF may also protect similar or non-overlapping populations of septal cholinergic neurons from atrophy and/or death. Preferably, the cells are fibroblasts which have been genetically engineered to produce recombinant human NGF.

Fornix lesions also cause behavioral deficits in the animal subjects of the model, most easily observed in tasks involving learning and memory. It has been reported that chronic administration of NGF to rats with fimbria-fornix lesions accelerates the animals' behavioral recovery (Wills et al. Behav. Brain Res., 17, pp. 17-24 (1985)). In the present invention, implantation of the polymer capsule containing NGF-secreting cells provides a practical way to deliver NGF continuously to the appropriate brain region of the lesioned animal. The capsules of the present invention offer a practical form of therapy and/or prophylactic treatment for Alzheimer's victims whose conditions may

be ameliorated by continuous delivery of NGF to specific brain regions.

The methods and compositions of this invention may be used for the treatment of age-related cognitive defects resulting from neural degeneration. Such treatment may augment cognitive performance, thus providing a symptomatic benefit. Alternatively, treatment may provide a neuroprotective effect, although not a symptomatic benefit. Age-related cognitive dysfunction and dementia in humans has been related to neuronal degeneration, especially of cholinergic basal forebrain neurons, and the decline of cortical and hippocampal levels of ChAT (Coyle et al., Science, 219, pp. 1184-90 (1983); Whitehouse et al., Science, 215, pp. 1237-39 (1982); Phelps et al., Neurobiol. Aging, 10, pp. 205-07 (1989); Gage et al., Neurobiol. Aging, 9, pp. 645-55 (1988)). Preclinical research attempting to produce a rodent model of dementia by selectively destroying cholinergic basal forebrain neurons has failed to produce large, lasting deficits in cognitive function. Aged rats exhibit neuropathology similar to that reported in aged and demented patients. The spatial-learning Morris water maze is extremely sensitive to the deleterious effects of these pathological processes (Morris, J. Neurosci. Meth., 11, pp. 47-60 (1984); Morris, Learning and Motivation, 12, pp. 239-60 (1981)). This task has been validated as a measure of age-related cognitive function -- the performance of aged rats in this task is not strongly related to their motor, sensorimotor or visual deficits, factors which confound other tests of learning and memory (Gamzu, Ann. NY Acad. Sci., 444, pp. 370-93 (1985)).

As a cognitive task which requires the development of a spatial map (Eichenbaum et al., J.

Neurosci., 10, pp. 3531-42 (1990)), the Morris water maze seems analogous to nonverbal tests of cognitive function that are especially sensitive to senescence and dementing disorders in the clinical setting. Therefore, this task seems to be valid for the assessment of potential new treatments for dementia. For example, several studies have reported that exogenous NGF improves Morris water maze performance in aged and learning-impaired rats. See, e.g., Fischer et al., Nature, 329, pp. 65-68 (1987); Fischer et al., J. Neurosci., 11, pp. 1889-1906 (1991).

Because NGF does not readily cross the blood brain barrier, its administration into the CNS requires the use of invasive procedures which compromise the integrity of the blood brain barrier. For example, in the rodent preclinical studies that demonstrated the potential efficacy of exogenous NGF, the NGF was administered with osmotic minipumps or through chronic intraventricular cannulae. Those techniques require repeated infusions into the brain, either through injections via the cannulae, or from pumps which must be replaced every time the reservoir is depleted. Every occasion in which the pump reservoir must be replaced or the injection syringe reinserted through the annulae represents another opportunity that contaminants might be introduced into the brain, which is especially susceptible to infection.

Even with the careful use of sterile procedures, there is risk of infection. It has been reported that even in intensive care units, intracerebroventricular catheters used to monitor intracranial pressure become infected with bacteria after about three days (Saffran, Perspectives in Biology and Medicine, 35, pp. 471-86 (1992). In addition to the risk of infection, there seems to be

some risk associated with the infusion procedure. Infusions into the ventricles have been reported to produce hydrocephalus (Saffran et al., Brain Research, 492, pp. 245-254 (1989)) and continuous infusions of solutions into the parenchyma is associated with necrosis.

Use of fetal tissue is clouded by ethical concerns and unencapsulated non-fetal cells may be rejected or produce tumors. In addition, tissue taken from fetal sources may be highly variable. By encapsulating NGF-producing cells, exogenous NGF can be supplied with a relatively low risk of infection, without the use of fetal tissue, and without the risk of tissue rejection or tumor development.

Finally, concerns have also been expressed about whether exogenous NGF at the doses previously used itself might prove harmful or toxic, perhaps even accelerating the neurodegenerative processes associated with Alzheimer's disease (Saffran, Perspectives in Biology and Medicine, 35, pp. 471-86 (1992). It has been suggested that exogenous NGF might accelerate tangle formation, initiate axon sprouting of perivascular sympathetic axons potentially leading to changes in cerebral blood flow, or remodel the projections of basal forebrain neurons in response to the exogenous NGF such that not-yet-affected basal forebrain neurons become dysfunctional and thus accelerating the dementing process (Saffran, Perspectives in Biology and Medicine, 35, pp. 471-86 (1992)).

According to one aspect of this invention, the beneficial effects of exogenous NGF for the treatment of age related cognitive defects, including Huntington's disease, Parkinson's disease, Alzheimer's and ALS, may be obtained with doses much lower than

previously reported to be effective for exogenous hNGF delivery.

Administration of the previously reported doses of NGF may have undesirable side effects including severe weight loss, pain, listlessness, hypophagia and recurrence of herpes infection.

According to this invention, capsular delivery of NGF, synthesized in vivo, to the brain ventricles, brain parenchyma, or other suitable CNS location, ranging from 1-1500 ng/day is desirable. The actual dosage of NGF, or other suitable factor, can be varied by implanting a fewer or greater number of capsules. We contemplate delivery of 1-1500, preferably 10-600, most preferably 50-500, ng NGF/human/day, for ventricular delivery and 1-1500, preferably 10-150, ng NGF/human/day for parenchymal delivery. These dosage ranges are significantly lower than those previously reported doses of NGF needed for CBF neuronal sparing/sprouting in rodent studies and in primate studies (17-350 μg/day), especially if the dosages are normalized to account for brain volume differences between rodents, primates and humans. Tuzynski et al., J. Neurosci., 10, pp. 3604-14 (1990); Koliatsos et al., Ann. Neurol., 30, pp. 831-840 (1991), Koliatsos et al., Experimental Neurol., 112, pp. 161-73 (1991); Dekker et al., Neuroscience, 60, pp. 299-309 (1994). In the one clinical patient evaluated, the dose of NGF delivered was 75 μg/day. (Olson et al., J. Neural Trans., 4, pp. 79-95 (1992)). In one embodiment, genetically-modified cells secreting human NGF (hNGF) are encapsulated in semipermeable membranes, and implanted intraventricularly or intraparenchymally in a suitable mammalian host, preferably a primate, most preferably a human.

In order that this invention may be better understood, the following examples are set forth. These examples are for purposes of illustration only, and are not to be construed as limiting the scope of this invention in any manner.

Examples

EXAMPLE 1: Encapsulated NGF-Secreting BHK Cells Implanted In The CNS of Adult Rats

Human ß-NGF expression and BHK cell line production

The human gene for ß-NGF coding for the complete amino acid sequence of the pre-pro form of NGF was subcloned behind the mouse metallothionein promoter in an expression construct that contains the mutant form of dihydrofolate reductase (see, e.g., Kaufman United States patent 4,470,461) driven by the SV40 promoter (Fig. 1).

Two human genomic clones coding for the 5' and 3' ends of the ß-NGF gene were purchased from ATCC (phbeta N8D8, phbeta N8B9). A 440 bp 5' Sca1-EcoR1 fragment from phbeta N8D8 was ligated to a 3' 2.0 kb EcoR1 fragment isolated from phbeta N8B9. The spliced NGF genomic sequence contained ~37 bp of the 3' end of the first intron, the double ATG sequence believed to be the protein translation start for pre-pro-NGF and the complete coding sequence and entire 3' untranslated region of the human gene (Hoyle et al., Neuron 10, pp. 1019-1034 (1993)). The combined 2.51 kb ß-NGF construct was subcloned into the DHFR based pNUT expression vector (Baetge et al., Proc. Natl. Acad. Sci. USA, 83, pp. 5454-5458 (1986)) immediately downstream from the mouse metallothionein-I promoter (-650 to +7) and the first intron of the rat insulin II gene (Palmiter R.D. et al., Proc. Natl. Acad. Sci. USA, 88, pp. 478-482 (1991)). The pNUT-ß-NGF construct (see

Fig. 1) was introduced into BHK cells using a standard calcium phosphate-mediated transfection method. Transfected BHK cells were selected in medium containing 200 μM methotrexate (Sigma) for 3-4 weeks and resistant cells were maintained as a polyclonal population either with or without 200 μM methotrexate.

Quantitation of NGF bioactivity

Nerve growth factor (NGF) causes a marked outgrowth of neurite processes in PC12 cells and as such provides a rapid and sensitive assay for NGF bioactivity. To test for the bioactivity of the NGF produced by the NGF-transfected BHK cells, conditioned medium (CM) from parental BHK cells (BHK-control) and BHK-NGF cells were added to the PC12A (Schweitzer and Kelly, J. Cell Biol., 101, pp. 667-676 (1985)) cells grown on 6 well standard tissue culture plates. As a control, 25 S mouse NGF was added to some of the wells to induce neurites (50 ng/ml). The PC12A cells were scored for neurites that were ≥ 3 times the length of the cell body diameter over a period of 1-4 days. NGF bioassays were also performed upon retrieval of implanted control and NGF-secreting, BHK cell-loaded, capsules by adding CM from the capsules to naive PC12A cells taken from capsules incubated with fresh medium for 24 hours. In all of the experiments, CM from BHK-NGF cells produced a robust neurite outgrowth in PC12A cells within 24 hours indicating that the NGF produced from the BHK cells was bioactive. BHK-controls showed no such capacity to elicit neurite outgrowth in the PC12A cells in parallel experiments.

To determine whether the neurite outgrowth was due to factors other than NGF, such as bFGF, we added an NGF blocking antibody (mouse anti-ß-NGF; Boehringer-Mannheim Cat. # 1008-220) in combination

with CM and recombinant hNGF. The addition of this blocking antibody fully inhibited neurite outgrowth from the PC12A cells in a dose-dependent fashion. Finally, we performed a set of experiments probing the BHK-control cell conditioned medium by ELISA for the presence of basic fibroblast growth factor (bFGF). Using a bFGF ELISA (Research and Diagnostics Systems, Quantikine™, human FGF basic ELISA kit; Burgess et al. Ann. Rev. Biochem, 58, p. 575 (1989)), we were unable to detect any bFGF in heavily conditioned medium from the BHK cells. Both of these experiments clearly indicate that the neurite outgrowth seen in the PC12A cells in response to BHK-NGF CM is due to the presence of secreted NGF.

NGF ELISA

The quantitation of NGF expression from the encapsulated and the unencapsulated BHK-NGF cells was performed as follows: All of the reagents were obtained from Boehringer Mannheim Biochemicals unless otherwise noted. Nunc-Immuno MaxiSorp ELISA plates were coated with 150 µl per well of anti-mouse-ß(2.5S) nerve growth factor at 1 ng/ml in coating buffer (1xPBS without $CACl_2$ and without $MgCl_2$/0.1% sodium azide; pH 9.6). The coated plates were incubated at 37°C for at least 2 hours or alternatively at 4°C overnight. The coating solution was withdrawn from the wells and the wells were washed three times with 300 µl wash buffer (50 mM Tris-HCl/200 mM NaCl/10 mM $CaCl_2$/1% Triton X-100/0.1% sodium azide; pH 7.0). The wells were then blocked with 300 µl of coating solution with 10 mg/ml of bovine serum albumin (BSA) at room temperature for at least 30 minutes. The wells were then washed three times with 300 µl wash buffer. Conditioned medium samples were diluted 1:1 in 2x sample buffer (the

sample buffer is the same as wash buffer, only with 2% BSA). 100 µl of the prepared samples were loaded into the wells. The plates were covered and then incubated for at least 2 hours at 37°C or overnight at 4°C. The solutions were removed from the wells by suction and washed three times with 300 µl of wash buffer. To each well, 100 µl of 4U/ml of anti-mouse-ß (2.5S) nerve growth factor-ß-gal conjugate was added. The plates were incubated at 37°C for at least 1 hour. The solutions were removed from the wells by suction and washed three times with 300 µl of wash buffer. Finally, 200 µl of chlorophenol red-ß-D-galactopyranoside substrate solution (40 mg CPRG in 100mM Hepes/150 mM NaCl/2 mM $MgCl_2$/0.1% sodium azide/1% BSA; pH 7.0) was added to the wells and incubated at 37°C. After approximately 30 minutes to one hour or after the color development was sufficient for photometric determination at 570 nm, the samples were analyzed on a plate reader and measured against recombinant NGF protein standards.

***c-fos* induction assay**

Qualitative *c-fos* induction elicited by NGF administration to PC12A cells was measured by an immunofluorescence assay. PC12A cells were plated at a density of 100,000 cells per ml on poly ornithine-treated glass coverslips (12 mm) and allowed to equilibrate for at least 24 hours in a 24-well plate. Cells were grown in the same medium as previously described in the section on neurite outgrowth bioassay.

To test for *c-fos* induction in the PC12A cells, conditioned medium from capsules containing BHK and BHK-NGF cells or recombinant human NGF (50 ng/ml) was added to the PC12A cells for 2 hours and allowed to incubate at 37ºC and 5% CO_2. Following this

incubation, the coverslips were fixed with 4% paraformaldehyde (in 0.1 M PBS, pH 7.4), washed 2 X with 10mM glycine in PBS, permeabilized with 1% triton X100 (in PBS for 10 minutes) and 1% nonidet P40 (in PBS for 10 minutes). The cells on coverslips were washed 3 X 5 minutes with PBS, blocked with 5% normal goat serum (NGS) in PBS for 1 hour and incubated in a rabbit polyclonal antiserum (Oncogene Science) raised against *c-fos* diluted 1:10 in 1% NGS in PBS for 3 hours. The coverslips were then washed 2 X 5 minutes with PBS and incubated with a fluorescein-conjugated goat anti-rabbit IgG antibody. Finally, the coverslips were washed 2X with PBS and mounted with Citifluor® antifadent and viewed by fluorescence microscopy. Fluorescence was monitored by microscopy and *c-fos* induction measured by the presence of fluorescently labeled nuclei.

Encapsulation procedure

Asymmetric single skin hollow fibers were cast from solutions of 12.5% poly (acrylonitrile vinyl chloride, i.e. PAN-PVC) copolymer in dimethyl sulfoxide (w/w). The fabrication process is known as phase inversion using a dry-wet (jet) spinning technique according to Cabasso, Encyclopedia of Chemical Technology, 12, pp. 492-517 (1980). After the spinning process, the hollow fibers were sterilely transferred into a distilled water bath containing 25% glycerol, which provides a method for keeping the pores intact during the drying procedure (Cabasso, 1980, supra). The fibers produced (XP 11) were a T1/2 membrane type, having an inner diameter of 450 ± 25 μm, a hydraulic permeability of 53 ml/(m^2 min mmHg), a BSA rejection coefficient of 88.7 ± 2.1%, an ovalbumin rejection coefficient of 82.0 ± 1.7%, and a glucose mass transfer

coefficient of about $8x10^{-4}$ cm/s. After drying, devices were fabricated by mounting a length of 6-7 mm dry hollow fiber, with a distal seal, onto a light-cured acrylate hub with a septal fixture at the proximal end which has loading access for cells to be injected into the lumen of the device. Glycerol was removed from the devices with 70% filter sterilized ethanol and placed in HBSS prior to the encapsulation procedure.

Cells were loaded into the prefabricated encapsulation devices as follows: either BHK-control cells or BHK-NGF cells were loaded into prefabricated devices at a density of approximately 10^7/ml. The BHK cell suspensions at a density of 2 x 10^7/ml were mixed 1:1 with physiologic Vitrogen® (Celtrix, Palo Alto, CA), and infused into the pre-fabricated devices through the septal access port. After infusing 2-2.5 µl of the cellular suspension, the septum was cracked off and the access port was sealed using a light-cured acrylate (Zeneca). BHK cell-loaded devices were maintained in a serum-free defined medium, PC1 (Hycor), for 4-5 days prior to implantation. After 3 or 4 days in vitro, the cell-loaded capsules were washed twice in HBSS, and placed in 1 ml of fresh medium to be analyzed for NGF by ELISA.

Long Term ß-NGF Expression in Adult Rat CNS

Our in vitro experiments demonstrated long-term, stable, high level expression of human ß-NGF in BHK cells. To determine if this long-term, stable expression could be achieved in vivo, we implanted capsules containing BHK cells into the CNS of adult Lewis rats.

Prior to implantation, conditioned medium (CM) taken from BHK-NGF and BHK-control capsules were subjected to neurite outgrowth assay for NGF as

described above. 0.5 ml of CM from the BHK-NGF cells was equivalent to 50 ng/ml of exogenously added NGF, in terms of the extens of neurite outgrowth, whereas CM from the BHK-control cells did not contain detectable levels of NGF.

The capsules were implanted into the striatum of adult Lewis rats for one, three and six month periods. Upon explanation, the capsules were tested for NGF production by the neurite outgrowth assay.

After 1, 3, and 6 months in vivo, the BHK-NGF loaded capsules were able to produce neurite outgrowth in PC12A cells equivalent to or greater than 50 ng/ml of NGF. No NGF activity was detectable in the CM from the BHK-control capsules. As shown in Table I, ELISA quantitation of the samples from the encapsulated BHK-NGF cells release up to about 20 ng/24 hr/capsule after 3 and 6 months in vivo.

Table I

NGF AS MEASURED BY ELISA IN ng/CAPSULE/24 HR

Condition	Pre-	Post-
3 mo. nv1 (BHK-NGF)	NA	17.1
3 mo. nv2 (BHK-NGF)	NA	13.1
3 mo. nv3 (BHK-CONTROL)	NA	0.1
6 mo. nv1 (BHK-NGF)	NA	21.6
6 mo. nv2 (BHK-NGF)	NA	2.8
6 mo. nv3 (BHK-CONTROL)	NA	0.1

NA = Not Available; no medium samples for NGF ELISA pre-implant

nv1, nv2 and nv3 represent naive animals

EXAMPLE 2: Fimbria-Fornix Lesion Study in Rats

To evaluate the ability of encapsulated NGF-secreting BHK cells (as described in Example 1) to release efficacious amounts of NGF in vivo, fimbria-fornix aspirative lesions were stereotaxically performed in 14 Lewis rats. Immediately after lesioning, a BHK-control or BHK-NGF loaded XP-11 device, as described in Example 1, was stereotaxically implanted into the lesion site.

Subjects

Adult male Lewis rats (Harlan Breeders, Indianapolis, IN) approximately 3 months old and weighing approximately 300 grams were used in the following studies. The animals were housed in groups of three in a temperature and humidity controlled colony room which was maintained on a 12 hour light/dark cycle with lights on at 0700 hours.

Stereotaxic surgery

Immediately prior to surgery, rats were anesthetized with an intramuscular injection of a ketamine, xylazine and acepromazine mixture and positioned in a Kopf stereotoxic instrument (see Emerich et al. 1992). A sagittal incision was made in the scalp and a craniotomy performed extending 2.0 mm posterior and 3.0 mm lateral from Bregma. An aspirative device with a 20 gauge tip was mounted to a stereotaxic frame (Kopf Instruments) and the medial parietal cortex, cingulate cortex, corpus callosum, dorsal hippocampus, dorsal thalamus and fimbria-fornix were aspirated by placing the suction tip 1.40 mm posterior to Bregma and lowering it immediately lateral to the sagittal sinus to a depth of 5.0 mm. The tip was then moved laterally in 0.5 mm increments until a

position of 3.0 mm lateral to Bregma was attained. Immediately following the aspiration, the rats were unilaterally implanted with either transfected (N = 8) or non-transfected BHK cell-containing capsules (N = 6) by placing the capsule within an 18 gauge Teflon catheter mounted to the stereotaxic frame. Each device measured 0.7 cm in length by 600 microns in diameter and contained approximately 15-25 x 10^3 cells.

A stainless steel obdurator was placed within the cannula, and the obdurator held in place while the outer cannula was raised to passively place the capsule within the previously prepared cavity. The stereotaxic coordinates for implantation were: 0.5 mm posterior to Bregma, 1.0 mm lateral to the sagittal suture and 7.5 mm below the cortical surface.

Histology

Animals were anesthetized 3 weeks following surgery and prepared for histological analysis. Animals were transcardially perfused, using a peristaltic pump, with the following: 20 ml saline (0.9%, room temperature), 120 ml of glutaraldehyde (0.1%), 500 ml 0.1% glutaraldehyde/4% paraformaldehyde, 300 ml of paraformaldehyde (4%), and finally 300 ml of 10% sucrose. All solutions were ice cold (4°C) and prepared in phosphate buffered saline (pH = 7.4) unless otherwise noted.

Brains were removed following fixation, placed in 25% buffered sucrose (pH = 7.4) and refrigerated for 24-48 hours. Tissue was cut at 20 μm intervals on a cryostat and mounted onto polylysine coated slides. Every 3rd section throughout the septum was saved and processed for choline acetyltransferase (ChAT) immunoreactivity according to the following protocol: (1) overnight incubation in PBS containing

0.8% Triton X-100 + 10% normal serum, (2) 48 hour incubation with primary antibody (goat antiserum to ChAT; Chemicon) at a dilution of 1:1000, (3) 6 x 5 minute rinses in PBS + 0.2% Triton X-100 followed by a 1.5 hour incubation in biotinylated secondary antibody (IgG), (4) 6 x 5 minute rinses in PBS + 0.2% Triton X-100, (5) incubation with Avidin-Biotin Complex (ABC, Vector elite) for 1.5 hours, (6) 3 x 5 minutes rinses in PBS, (7) 5 minute rinse in distilled water, (8) incubation with 3,3-diaminobenzidine (DAB) (0.05%) + 2% nickel ammonium sulfate dissolved in 0.1% Tris buffer for 5 minutes followed by hydrogen peroxide (0.01%) for 5 minutes, (9) the reaction was terminated by 3 x 1 minute rinses in PBS.

Sections were mounted, dehydrated and coverslipped. Adjacent sections were stained for hematoxylin and eosin (H+E). To verify the extent of lesion produced following aspirations of the fimbria-fornix, every 10th section was taken throughout the hippocampus and stained for acetylcholinesterase according to the method of Van Ootegan et al. (Brain Res. Bull., 12, pp. 543-553 (1984)). For quantification of cholinergic cell loss, ChAT-positive neurons were counted in the medial septum and vertical limb of the diagonal band at a magnification of 10X. Representative sections (3 per brain) located approximately 0.7, 0.5 and 0.2 mm anterior to Bregma from each animal were used for this analysis.

NGF Release Results

At 3½ weeks post implantation the animals were anesthetized and the devices retrieved by gently pulling the silicone tethers. Each capsule was incubated for 24 hours in 1 ml of medium and CM was assayed for NGF by ELISA. Pre- and post-

transplantation NGF secretion levels are listed in Table II.

Table II

NGF AS MEASURED BY ELISA IN ng/CAPSULE/24 HR

Condition	Pre-	± SD	Post-	± SD
F/F UNILAT (n=4)	27.2	2.5	5.2	0.9
CONTROLS (n=6)	0.1	0.05	0.15	0.1

Histological sections taken through the hippocampus and septum of all animals were examined by immunocytochemistry for acetylcholinesterase (AchE) in the hippocampus and choline acetyltransferase (ChAT) in the septum. AchE immunoreactivity in the hippocampus was used as a second indicator of lesion completeness and ChAT-immunoreactivity in the septum provided evidence for cholinergic cell body sparing or atrophy as a result of transplant and lesions. A representative comparison of AchE immunostaining in the hippocampus of the lesioned-vs-control side demonstrated nearly complete loss of AchE afferents to the lesioned side.

Total ChAT-positive neurons remaining in the septum 3½ weeks post-lesion in BHK-control and BHK-NGF implanted animals were counted and the combined results are shown in Fig. 2. Quantitation of ChAT-positive neurons revealed that with BHK-control capsules, only 15±3% of the neurons remained positive on the lesioned side of the brain compared with the non-lesioned side, whereas with the BHK-NGF capsules, 90±5% of the cholineric neurons were immunopositive for ChAT. Encapsulated cell survival was equivalent between the BHK-control and BHK-hNGF cell-loaded capsules (data not shown).

EXAMPLE 3: Primate Fornix Lesion

Example 2 demonstrated that the chronic delivery of hNGF from the BHK cells of this invention into the lateral ventricle of adult rats with fimbria-fornix lesions protects the medial septal cholinergic neurons that otherwise would have died as a result of the lesion. Similar experiments are described here performed in non-human primates. Lesioning of the fornix in monkeys (*Cebus apella*) is described in (Kordower and Fiandaca, J. Comp. Neurol., 298, pp. 443, (1990)).

Adult cynomolgous monkeys were used in these lesion experiments. After the unilateral lesion was complete, 5 XP-11 capsule devices, as described in Example 1, (identified by number) were manually placed within the lateral ventricle adjacent to the medial septum, ipsilateral to the lesion. As controls, some animals were implanted with devices loaded with BHK-control cells. Following the implantation procedure, the surgical sites were closed and the animal were allowed to recover and were closely monitored for 3½ weeks. Following the 3½ week survival period, the numbered capsules were carefully removed and placed in standard cell culture medium and then reassayed by ELISA for NGF release as they were pre-implantation (Table III). The animals were then sacrificed by perfusing with aldehyde fixatives and the brains were sectioned and processed for histochemical and immunohistochemical procedures.

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(54) Title: MEDIUM FOR LONG-TERM PROLIFERATION AND DEVELOPMENT OF CELLS

(57) Abstract

A serum-free or serum-depleted medium for the short- and long-term proliferation and development of cells, particularly hematopoietic cells and bone marrow stromal cells, the medium comprising cell proliferation and development effective amounts of: a standard culture medium such as Iscove's modified Dulbecco's medium; serum albumin; transferrin; a source of lipids and fatty acids; cholesterol; a reducing agent; pyruvate; a glucocorticoid (when the cells to be cultured are hematopoietic cells); nucleosides for synthesis of DNA and RNA; growth factors that stimulate the proliferation and development of stromal cells and cells from a variety of tissues or organs, such as epidermal growth factor, fibroblast growth factor, platelet derived growth factor, and insulin; and extracellular matrix materials, such as collagen, fibronectin, and laminin.

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MEDIUM FOR LONG-TERM PROLIFERATION AND DEVELOPMENT OF CELLS

BACKGROUND OF THE INVENTION

I. Field of the Invention

This invention relates to serum-free or serum-depleted culture media for supporting the proliferation and development of cells. More particularly, it is directed to culture media for the long-term growth of hematopoietic cells and the stromal cells which support their growth. It is also directed to methods for culturing mammalian cells from different tissues and organs using such media. The media and methods enable the maintenance of cell growth and development for up to several months.

II. Description of Background and Related Art

One aspect of the present invention is a medium for culturing cells, particularly mammalian cells in the absence of or substantial depletion of serum, whereby the medium is chemically well defined, while still providing long-term support of development and proliferation of such cells.

In the past, many types of mammalian cells have been isolated and attempts have been made to propagate the cells in culture for further study or use. The media used for such culture have typically included sources of nutrients, adhesion factors and growth factors required for cell proliferation and development, many provided by horse, bovine or calf serum. However, the use of serum to support cell culture has been problematic since it does not provide physiological conditions. Indeed, the only time a cell would see the

sort of serum concentration normally used for cell culture would be at the site of a wound, where the blood is clotting. The use of serum is further complicated by high cost and the presence of undefined components in the serum which may vary from sample to sample. The presence or absence of these undefined components has led to inconsistent results in the culture of cells and lack of control over the culturing process. For example, a particular serum sample may introduce particular components into the culture medium that inhibit the growth of the cells.

In response to problems associated with the presence of serum, a variety of serum-free media have been produced in the past. See for example:

1) Barnes, D. & Sato, G. Serum free cell culture. A unifying approach. Cell. 1980; 22: 649;

2) Cormier, F., Ponting, I.L.O., Heyworth, C.M. & Dexter, T.M. Serum-free culture of enriched murine haemopoietic stem cells I: Effect of haemopoietic growth factors on proliferation. Growth Factors. 1991; 4: 157-164;

3) Deslex, S., Negrel, R. & Ailhaud, G. Development of a chemically defined serum-free medium for differentiation of rat adipose precursor cells. Experimental Cell Research. 1987; 168: 15-30;

4) Drouet, X., Douay, L., Giarratana, M.C., Baillou, C.L. Gorin, N.C., Salmon, C.H. & Najman, A. Human long-term bone marrow culture in serum-free medium. British Journal of Haematology. 1989; 73: 143-147;

5) Kumar, R.K., O'Grady, R., Li, W., Smith, L.W., Rhodes, G.C. Primary culture of adult mouse lung

fibroblasts in serum-free medium: responses to growth factors. Experimental Cell Research. 1991; 193: 398-404;

6) Migliaccio, G. Migliaccio, A.R. & Adamson, J.W. The biology of hematopoietic growth factors: Studies in vitro under serum-deprived conditions. Experimental Hematology 1990; 18: 1049-1055; and

7) Ponting, I.L.O., Heyworth, C.M., Cormier, F. & Dexter, T.M. Serum-free culture of enriched murine haemopoietic stem cells II: Effects of growth factors and haemin on development. Growth Factors. 1991; 4: 165-173.

As described in a number of the cited articles, there already exist methods for the short-term (up to 3-4 weeks) serum-free culture of hematopoietic progenitor cells, using added hematopoietic growth factors as the proliferative and developmental stimuli. However, the development of ideal serum-free media to study the long-term culture of hematopoietic cells has proven to be particularly difficult.

Prior to the invention described herein, mammalian long-term hematopoiesis has been studied *in vitro* mainly using serum supplemented bone marrow culture systems which appear to closely mimic some of the processes that occur *in vivo*. The first of these was described by Dexter, T.M., *et al.*, J. Cell. Physiol., 91: 335-344 (1977) and involves the formation of an adherent layer of murine bone marrow stromal cells, which included endothelial cells, fibroblasts, adipocytes and macrophages. This cell layer was required to support the hematopoietic cells, probably by providing a physical adhesive matrix as well as the

correct cell to cell signalling, including the necessary growth factors for hematopoietic cell proliferation and development.

The hematopoiesis which takes place in these Dexter cultures occurs in intimate contact with the stromal cell layer. In many cases the stem cells and progenitor cells proliferate under the stromal cells forming foci called cobblestone regions due to their characteristic appearance under phase microscopy. As the hematopoietic cells mature, many of them migrate to the uppermost surface of the stromal cells. Further maturation to a fully differentiated state results in release of the hematopoietic cells into the surrounding medium where they are removed by the bi-weekly feeding.

This culture system enabled the maintenance of hematopoiesis for several months, with the constant production of progenitor cells. Soon after, a similar system was developed for human cells (Gartner, S. and Kaplan, H.S., Proc. Natl. Acad. Sci. USA, 77: 4756 - 4759 (1980)).

One important drawback of the Dexter method was that it mainly allowed the development of macrophages and neutrophils, at the expense of other cell types normally produced by the bone marrow, particularly B lymphocytes. A method for the long-term production of B lymphocytes was discovered by Whitlock, C.A. and Witte, O.N., Proc. Natl. Acad. Sci. USA 79: 3608 - 3612 (1982). This system differed from that described by Dexter, *et al.* in that the horse serum was replaced with fetal calf serum and hydrocortisone was replaced with 2-mercaptoethanol. However, this system precluded the development of hematopoietic lineages other than B lymphocytes. The reason(s) for the differences in the development that occurs in these two culture systems, and why they do not mimic all aspects

of bone marrow hematopoiesis, is unknown but is possibly a reflection of the use of serum.

In spite of the many attempts that have been made to produce an ideal, chemically controlled medium for culturing cells, especially mammalian hematopoietic cells, a number of shortcomings have persisted in such media. For example, prior media do not truly enable long-term culture of such cells. In accordance with the present invention, "long-term" is defined as greater than or equal to approximately eight weeks of continuous proliferation and development, including generation of new progenitor cells in the medium. The media of the present invention can produce up to three to five months of continuous growth and development in culture. There have been no prior serum-free media that have been able to achieve such long-term culture of mammalian hematopoietic cells.

The following is a brief description of some of the additional related prior art:

Cormier, et al. Growth Factors. 1991; 4: 157-164, relates to serum-free culture of enriched murine hematopoietic stem cells and focuses on the effect of certain growth factors on proliferation. However, the medium disclosed in this publication contains different components in different relative proportions as compared to the present invention, and was unable to achieve any long-term growth (as defined herein), in contrast to the present media.

Dexter, T.M., et al., J. Cell. Physiol. 91: 335-344 (1977), discloses the classical Dexter long-term bone marrow culture method. It is notable that this method contains serum, unlike the present invention. As a result its use is restricted, as already described, to

certain mouse strains and even with such strains the growth is not as pronounced as in the current invention.

Dexter, T.M., et al., Long-Term Bone Marrow Culture. New York: Alan R. Liss: 57-96, is a review of long-term marrow culture techniques and media. None of the techniques or media disclosed in this reference contain the same group of components as the present invention.

Drouet, X., *et al.* Brit. J. of Haem. 73: 143-147 (1989), disclose a human long-term bone marrow culture medium that is serum-free. However, the medium disclosed therein is not capable of achieving the same degree of long-term culture as in the present invention, growth being maintained for only 3-4 weeks. Furthermore, unlike the media of the present invention, those discussed by Drouet, *et al.* do not involve stromal cells.

Whitlock, C.A., & Witte, O.N. P.N.A.S. 79: 3608-3612 (1982), relates to long-term culture of B lymphocytes and their precursors from murine bone marrow. This is the famous Witte-Whitlock culture technique. However, again, the media disclosed in this paper are substantially different from those claimed in the present invention, in that they contain serum and can only support B lymphocyte growth.

Teofili, L., *et al.* Ann. Hematol. 65: 22-25 (1992), describes a serum-free culture system for the growth of human hematopoietic progenitor cells using a preformed extracellular matrix. However, this method does not produce long-term growth, progenitor cells only being maintained in reasonable numbers for 3 weeks. In addition, this method does not use growth factors to

support the growth of the bone marrow stromal cells and does not use purified extracellular matrix materials for cell adherence. As a result it does not support the growth of cells from a wide variety of tissues and organs.

In spite of the above prior art, there remains a need to produce chemically defined media for long-term culture of cells. Ideally, such media should be simple to prepare, contain chemically defined ingredients, be economical to produce, and achieve optimal long-term growth and development of cells.

Accordingly, it is an object of the present invention to provide serum-free media for long-term maintenance of proliferation and development of cells, especially of hematopoietic origin.

A further object of the present invention is to provide methods for both short and long-term culture of cells in a chemically defined medium.

Another object of this invention is to provide a method for stimulating the proliferation and/or development of early progenitor cells for bone marrow transplants and/or gene transfer into these cells for gene therapy procedures.

These and other objects of the invention as will hereinafter be described in greater detail will be apparent to one of ordinary skill in the art.

III. Summary of the Invention

In accordance with the objects of the present invention, there is provided a medium for long-term proliferation and development of cells, which comprises cell growth and development-effective amounts of:

(a) a standard culture medium such as Iscove's modified Dulbecco's medium (IMDM), RPMI, DMEM, Fischer's, alpha medium, Leibovitz's L-15, NCTC, F-10, MEM and McCoy's;

(b) serum albumin;

(c) transferrin;

(d) a source of lipids and fatty acids;

(e) cholesterol;

(f) a reducing agent;

(g) pyruvate;

(h) nucleosides for synthesis of DNA and RNA;

(i) one or more growth factors that stimulate the proliferation and development of stromal cells and/or cells from a variety of organs and tissues (preferable mammalian), such as epidermal growth factor, basic fibroblast growth factor, platelet derived growth factor, and insulin; and

(j) one or more extracellular matrix materials;

wherein said medium is serum-free or serum-depleted.

An optional component of the serum-free medium which is used in cultures where neutrophil development and proliferation is desired (e.g., long-term bone marrow cultures) is:

(k) a glucocorticoid such as hydrocortisone, cortisol, dexamethasone or other structurally related, natural or synthetic molecule.

Another optional component of the serum-free or serum-depleted medium which may be added separately to the medium when the cells to be supported are hematopoietic cells is:

(l) stromal cells, which provide a supporting network for hematopoietic cells, as described below in greater detail.

In an exemplary embodiment of the present invention, particularly suited for growth of neutrophils, the cell culture medium comprises the following components in the indicated concentrations:

Component	Suitable Conc.
Iscove's Modified Dulbecco's Medium	0.7 - 1.17 x
Bovine serum albumin	>1 mg/ml
Bovine transferrin	> 25 µg/ml
Soybean lipids	>5 µg/ml
Cholesterol	>1 µg/ml
2-mercaptoethanol	10 - 400 µM
Sodium pyruvate	>20 µg/ml
Hydrocortisone	0.05 - 5 µM
Nucleosides	1 - 100 µg/ml
Epidermal growth factor	> 0.5 ng/ml
Fibroblast growth factor	> 0.1 ng/ml

Component	Suitable Conc.
Platelet-derived growth factor	> 0.5 ng/ml
Insulin	> 0.5 µg/ml
Collagen IV	> 1 µg/cm^2
Fibronectin	> 0.2 µg/cm^2

In a preferred embodiment of the present invention, the cell culture medium used to grow cells from different organs or tissues is as already described except that it does not contain any hydrocortisone, or other related glucocorticoid.

In another preferred embodiment, hydrocortisone or another glucocorticoid is not present in the medium and stimulation of the hematopoietic progenitor cells by the growth factor CSF-1, a developmental and proliferative stimulus for macrophages, is also prevented. This can be achieved in a number of ways. A mutant strain of mice can be used in which the CSF-1 gene (B6C3Fe-a/a-op/op mouse strain) or the gene for its receptor or part of the CSF-1 receptor signalling pathway is inactivated, so that no functional protein is produced. A variety of inhibitors of CSF-1 activity could also be employed. For example, neutralizing antibodies against CSF-1 or its receptor could be used to prevent stimulation. In addition it is possible to use antisense oligomers (short sequences of single stranded nucleic acids) to prevent the expression of CSF-1, its receptor or some part of the signal transduction mechanism. The hematopoietic development in these cultures is not restricted to neutrophils and macrophages, but also includes other cell types such as lymphoid, mast cell, megakaryocyte and erythroid cells.

In a particularly preferred embodiment, the cells to be cultured are mammalian in origin.

In a further particularly preferred embodiment, the cells are hematopoietic or lymphopoietic in origin.

IV. Brief Description of the Drawings

FIGURE 1 depicts a comparison of the current invention (LTBMC-SFM) and prior serum-free media, in terms of the number of bone marrow adherent cells (stromal and hematopoietic cells) after 7 days. Note that the present media gave very good hematopoiesis after 2 weeks, whereas hematopoiesis was not observed in any of the prior media tested.

FIGURE 2 depicts the effect of removing one of the groups of serum-free medium components on the establishment of long-term bone marrow cultures as indicated by the adherent cell counts after 7 days. Error bars show standard deviations.

FIGURE 3 depicts the effect of adding serum-free medium components to serum supplemented "Dexter" cultures (DEX), including growth factors (+ GFs), Iscove's medium (+ IMDM) instead of Fischer's medium, extracellular matrix materials (ECM), additional nutrients (NUTS), and a temperature of 37°C. rather than 33°C. The cultures SFM + 37°C + DEX were set up under standard serum-free medium conditions, but with horse serum. Error bars show standard deviations.

FIGURE 4 depicts the relative number of adherent cells (a combination of bone marrow stromal and hematopoietic cells) in serum supplemented and serum-free media after 7 days of culture. The importance of extracellular matrix materials such as collagen and fibronectin in serum-free cultures is also shown. Fibronectin is at a conc. of 2 $\mu g/cm^2$. Error bars show standard deviations.

FIGURE 5 depicts the long-term hematopoiesis which occurs using the current invention, as indicated by the total number of non - adherent hematopoietic cells (mainly mature cells) produced over an 11 week period, ± standard deviation.

FIGURE 6 depicts the long-term hematopoiesis which occurs using the current invention, as indicated by the number of non-adherent hematopoietic progenitor cells (colony forming cells) produced over a 10 week period, ± standard deviation.

FIGURE 7 depicts the production of non-adherent hematopoietic cells after 21 days from serum-free long-term bone marrow cultures when using different strains of mice. C3H, C57Bl/6, Balb/c, and DBA/2 are inbred strains of mice; CD-1 is an outbred strain of mouse; while $B6D2F_1$ (BDF1) and $B6C3F_1$ are hybrids (female C57Bl/6 x male DBA/2 and female C57Bl/6 x male C3H respectively). Error bars show standard errors.

FIGURE 8 shows a table which depicts the morphology of the non-adherent hematopoietic cells produced in long-term bone marrow cultures of normal (+/+) mutant (op/op) mice in the presence or absence of hydrocortisone (HC).

V. Detailed Description of the Invention

A. Comparison of the Invention with Prior Media

The essential reason why prior serum-free media have failed to give long-term growth of normal hematopoietic cells is that they have not contained the correct combination of ingredients in the proper concentration ranges to support the growth of a wide range of cell types, including hematopoietic cells and stromal cells. This has resulted in many prior serum-free media being limited to supporting the growth of

cell lines, rather than the more difficult to maintain, normal primary cells (i.e., those cells that come directly from an animal). Those media that are capable of maintaining the growth of normal cells only do so for one or a few cell types, and do not provide permissive growth conditions for the long-term culture of hematopoietic cells and stromal cells. In contrast, media of the present invention have the unique ability to support the growth of very different cells from a wide variety of tissues and organs, including primary (normal) cells and cell lines. This ability enables the long-term growth of both the hematopoietic progenitor cells as well as the bone marrow stromal cells (adipocytes, macrophages, endothelial cells and fibroblasts) which are essential for supporting the growth of the hematopoietic cells. This is an ability that prior serum-free media do not possess.

Another shortcoming of prior serum-free media was their species specificity; in contrast, the media of the current invention can support the growth of both mouse and human cells, and they are expected to support the growth and development of cells from other species as well.

While the current invention is a dramatic improvement over prior serum-free media, it is also an improvement over standard serum-supplemented bone marrow cultures. For example, present techniques are limited in the strains of mice from which long-term hematopoiesis can be obtained, whereas no such limitation exists for the current invention, hematopoiesis being obtained from every strain tested. Furthermore, the rate of bone marrow stromal and hematopoietic cell growth under the conditions described in the present invention greatly exceed those in serum-supplemented cultures due to the improvement of numerous aspects of the culture conditions, e.g. adherence, temperature, nutrients and

growth factors. In this way, the 4-5 week long period required for classical serum- supplemented long-term bone marrow cultures to produce significant numbers of hematopoietic cells can be reduced to just around 2 weeks.

A further major advantage of the media of this invention over prior methods for the long-term culture of hematopoietic progenitor cells which use serum is the control which it allows in manipulating the proliferation and development of the hematopoietic cells. The reason for this is that all of the ingredients and their concentrations in the culture system are known and can therefore be controlled. This contrasts to serum supplemented cultures where the contents of the serum are generally unknown, and where the concentrations of the known components are similarly undefined.

For example, by decreasing the concentration of one of the culture ingredients it is possible to efficiently make the culture conditions sub-optimal for growth, thus reducing the number of hematopoietic and/or stromal cells produced.

In a similar way changes in hematopoietic stem cell development can be induced by the removal of the serum-free medium ingredients, hydrocortisone, which is required for optimal growth of neutrophilic cells; as well as the removal of the growth factor CSF-1, a stimulus for macrophage growth, normally produced by the stromal cells in the culture. These alterations in the culture result in not only neutrophil and macrophage development occurring, but also lymphoid, mast cell and possibly erythroid and megakaryocyte development as well. This type of varied development is far more representative of that which occurs in the bone marrow *in vivo*, and is not observed in any of the prior

techniques for the long-term culture of hematopoietic cells.

The use of a defined culture system also makes possible the precise determination of the effect of a known molecule on the cells. Such an analysis is not possible using serum-supplemented cultures where the test molecule may already be present at an unknown concentration.

B. Definitions

In general the "medium" and "media" as used in connection with the present invention are solutions containing growth factors and nutrients, which are used to support the growth and development of cells.

By "long-term" is meant continuous growth and development of the cells being cultured, as well as generation of progenitor cells, for a time period of at least about 8 weeks and up to or surpassing about 12 - 20 weeks. Preferably, "long-term" means 8-12 weeks.

The "cells" that may be supported (i.e., grown and developed/differentiated) by the media of the present invention are preferably mammalian cells, such as: human, monkey, bovine, ovine, equine, and murine. They are preferably, human or murine cells. Cells from other sources as well as from other mammalian species are also contemplated as would be apparent to one of ordinary skill in the art.

Such cells may be derived from a variety of "tissues," such as lung, liver, kidney, thymus, thyroid, heart, brain, and the like.

Particular examples of normal mammalian cells that may be cultured in the media of the present invention are fibroblasts, endothelial cells, adipocytes, glial cells, neuronal cells, myoblasts, epithelial cells (which may be from a wide range of

different tissues), hepatocytes, osteoclasts, and heart muscle cells. The culture media also support a wide range of cell lines including immortalized versions of those already listed; examples of others include bone marrow stromal cell lines, embryonic stem cell lines (such as: D3, E3, SQ1.2S8, MBL-1, 632, and CCEG2), embryonic carcinoma cell lines (such as PGC3), melanoma, mammary, and pituitary cell lines.

Preferred cells of the present invention to be cultured are hematopoietic and lymphopoietic cells which are preferably obtained from the spleen, fetal liver, peripheral blood, umbilical cord or bone marrow.

In general, the present culture media have successfully supported the growth of every cell type so far tested and it is anticipated that it will support the growth of many mammalian cell types.

The term "stromal cells" is used to refer to those cells of the bone marrow which form a supporting network for hematopoietic cells *in vivo* and *in vitro*. In this respect they support the hematopoietic cells both physically in providing sites of attachment, as well as biologically in providing the cytokines which these cells require for their growth. In culture they form an adherent layer and include endothelial cells, macrophages, fibroblasts and pre-adipocytes/adipocytes. Accordingly, when the cells to be supported are hematopoietic cells, stromal cells will preferably also be included either as part of the medium, or added to the medium (preferably, 10^4 - 10^7 stromal cells per culture) during the time the hematopoietic cells are present in the medium.

The term "amino acid" refers to all naturally occurring alpha amino acids in both their D and L stereoisomeric forms, and their analogs and derivatives. An analog is defined as a substitution of an atom in the amino acid with a different atom that usually has

similar properties. A derivative is defined as an amino acid that has another molecule or atom attached to it. Derivatives would include, for example, acetylation of an amino group, amination of a carboxyl group, or oxidation of the sulfur residues of two cysteine molecules to form cystine.

The amino acids are identified by either the standard single-letter or three-letter designations.

"Serum-free" is used herein to mean that substantially all serum is excluded from the medium (for example 0-0.49 %). Further to this is the term "serum-depleted" which means that up to about 5% of serum could be added to the medium (for example 0.5-5 %). Preferably less than 3% of the medium will be derived from serum, particularly preferably less than 0.5%. In contrast, serum-containing media of the prior art have typically included >5-40% serum.

By "cell culture effective amount" is meant an amount that is capable of producing long-term cell proliferation and development. Such amounts may be determined for a given constituent by experimentation wherein varying concentrations of a given constituent are added to cell growth media, and the extent and duration of the cell proliferation and development is determined by methods known to a skilled cell culturist. Preferred ranges and examples of such amounts are provided throughout the specification.

C. Description of Culture Media

An important aspect of the present invention is the discovery that combinations of ingredients in the proper amounts can support both short- and long-term growth (i.e. proliferation) and development of mammalian cells, particularly hematopoietic cells.

In a general sense, there are three primary types of materials included in the present media:

Extracellular matrix materials

These are involved in adhesion of cells, such as to the culture surface. They are typically included in a concentration of 0.2 - 100 $\mu g/cm^2$.

Examples of extracellular matrix materials from mammalian sources (bovine or human, though preferably murine) that are preferred are: a source of collagen (preferably collagen I and collagen IV), fibronectin, vitronectin and laminin. Especially preferred are:

- Mouse collagen IV, which can be used at a concentration of greater than 1 $\mu g/cm^2$, preferably from about 2 - 100 $\mu g/cm^2$, and more preferably at about 5$\mu g/cm^2$.
- Mouse fibronectin, which can be used at a concentration of greater than 0.2 $\mu g/cm^2$, preferably from about 0.5 - 100 $\mu g/cm^2$, and more preferably at about 2 $\mu g/cm^2$.

However, less pure sources of extracellular matrix can also be used, such as MATRIGEL, which is a complex mixture of matrix and associated molecules. Matrix secreted by a previously adherent cell can also be used. The cell is grown on the plastic culture surface and secretes extracellular matrix, removal of these cells then provides a layer of extracellular matrix to which a second cell type can adhere.

Growth factors

These are molecules involved in stimulating proliferation of the stromal cells or other cells from a variety of tissues that are desired to be supported by the media. In the case of bone marrow stromal cells they produce growth factors for the hematopoietic cells in the culture, e.g. GM-CSF, G-CSF, SCF, IL-1, CSF-1, IL-6,

and a variety of others. In cases where a purified population of hematopoietic cells is cultured using the serum-free medium these growth factors have to be added (typically in concentrations of 0.5 ng/ml - 100 µg/ml, depending on the growth factor and the cells involved), since the stromal cells will have been removed.

The choice of growth factors required to support the growth and development of particular types of cells will vary depending on the cell type, and will be readily determinable by those skilled in the art of cell biology and culture. Growth factors for the stromal and tissue cells that are particularly useful in accordance with the present invention, and to which additional specific growth factors can always be added as desired or necessary for growth include:

- Recombinant human epidermal growth factor (EGF), which can be used at a concentration of greater than 0.5 ng/ml, preferably from about 5 - 200 ng/ml, and more preferably at about 15 ng/ml.
- Recombinant human basic fibroblast growth factor (bFGF), which can be used at a concentration of greater than 0.1 ng/ml, preferably from about 0.5 - 40 ng/ml, and more preferably at about 2 ng/ml.
- Recombinant human platelet derived growth factor (PDGF) (preferably the B-B isoform), which can be used at a concentration of greater than 0.5 ng/ml, preferably from about 2 - 200 ng/ml, and more preferably at about 10 ng/ml.
- Insulin (human or preferably from bovine pancreas) can be used at a concentration of greater than 0.5 µg/ml, preferably from about 2 - 100 µg/ml, and more preferably at about 10 µg/ml.

Less pure sources of growth factors such as conditioned media from cell lines producing these growth factors can also be used, but do not give such a defined

culture. In addition it is possible to use growth factors from other species, e.g. bovine and mouse.

Nutrients

These are molecules involved in normal metabolic functions of the cultured cells.

Nutrients and metabolic additives useful in the media of the present invention include a standard culture medium (described below); serum albumin (preferably bovine or human); transferrin (preferably bovine or human); a source of lipids and fatty acids useful in cell growth and development (preferably soybean lipids); cholesterol; a reducing agent (preferably 2-mercaptoethanol or monothioglycerol); pyruvate (preferably sodium pyruvate); a glucocorticoid (preferably, a cortisone derivative such as hydrocortisone 21-hemisuccinate, particularly preferably the sodium salt); and nucleosides.

The following is a more detailed description of some of the individual components of the nutrients in the media of the present invention:

- The "standard culture media" which may be employed in accordance with the present invention are standard culture media for growing cells that typically provide at least one component from the following categories:

(a) an energy source, usually in the form of a carbohydrate such as glucose;

(b) substantially all essential and non-essential amino acids, usually the basic set of twenty amino acids, plus cystine instead of cysteine;

(c) vitamins and/or other cell-growth supporting organic compounds required at low concentrations;

(d) a buffering agent such as HEPES, Tris, or MOPS (preferably HEPES), which act to stabilize

the hydrogen ion concentration and therefore the pH of the solution by neutralizing, within limits, both acids and bases;

(e) inorganic salts and trace elements, where trace elements are defined as inorganic compounds or naturally occurring elements that are typically required at very low concentrations, usually in the micromolar range (e.g., 1 - 1000 μM).

Particular examples of the standard culture media which are useful in accordance with the present invention are: RPMI, DMEM, MEM, Leibovitz's L-15, Fischer's, F-10, alpha medium, NCTC and McCoy's, but preferably Iscove's modified Dulbecco's medium (IMDM); Iscove, N.N. & Melchers, F. J. Exp. Med. 147, 923 (1978).

In accordance with this invention, the standard culture medium is supplemented with particular components from the following categories:

The "serum albumin" of the present invention may be obtained from any mammalian source, such as human, monkey, bovine, ovine, equine, murine. Preferably the source of serum albumin is human or bovine. The serum albumin can be used at a concentration of greater than 1 mg/ml, preferably from about 3 - 50 mg/ml, and more preferably at about 10 mg/ml. It is notable that although the present media are generally serum-free, certain serum components which are chemically well defined and highly purified (>95% pure), such as serum albumin, may be included.

Transferrin is another protein component of the media set forth herein which is also preferably of mammalian origin, particularly preferably human or bovine. This component can be incorporated at greater than 25 μg/ml, preferably from about 25 - 1000 μg/ml, and more preferably at about 100 - 300 μg/ml.

"A source of lipids and fatty acids" means any of a variety of such sources. For example, crude extracts of lipids may be used to supplement the media of this invention. The lipid extract may be obtained from any biological source, although one preferred source is plant material, and particularly soybeans (soybean lipid extract may be purchased from Boehringer-Mannheim). Typically, the extract will contain from about twenty percent to about ninety-five percent lipid, the majority of which will be phospholipid, the remainder being fatty acids, with traces of sterol. The extract may be added to the cell culture medium at a concentration of greater than about 5 µg per ml, preferably from about 5 µg per ml to about 100 µg per ml, and more preferably 5 µg per ml to about 50 µg per ml.

The addition of a molecule capable of regulating the cell plasma-membrane fluidity is an important component. Preferably, this invention employs the lipid cholesterol at a concentration of greater than about 1 µg/ml, or preferably from 3 - 30 µg/ml, or more preferably at about 7.8 µg/ml.

The term "a reducing agent" means any reducing agent that is compatible with a cell culture medium. Preferred examples are: 2-mercaptoethanol or monothioglycerol. Generally, the reducing agent is present in a concentration of about 10 to 400 µM, preferably 30 to 300 µM, and particularly preferably, about 100 µM.

"Pyruvate" refers to any cell-culture compatible salt of pyruvic acid, preferable sodium or potassium pyruvate. Pyrucic acid itself may also be added, forming a salt to some degree in situ. Pyruvate is required for general metabolic processes as a potential source of energy and for synthesizing more complex molecules. Generally, the pyruvate is present

in a concentration of 20 µg/ml, preferably 30 to 500 µg/ml, and particularly preferably, about 110 µg/ml.

The term "nucleosides" refers to naturally occurring nucleosides that form the basis for synthesis of DNA and RNA. Such nucleosides include the following: adenosine, guanosine, cytidine, uridine, 2'-deoxyadenosine, 2'-deoxyguanosine, 2'-deoxycytidine and thymidine. Phosphorylated (mono, di, or tri) analogues could also be used. The nucleosides may be added to the cell culture medium at a concentration of about 1 to 100 µg/ml, preferably from about 5 to 30 µg/ml and particularly preferably about 10 µg/ml.

The growth factors epidermal growth factor, fibroblast growth factor, platelet-derived growth factor and/or insulin which have already been discussed in detail in section B are prferably all included in the standard medium.

A component which is not required in all embodiments of the invention is a natural or synthetic corticoid such as the cortisone derivatives: prednisone, dexamethasone or preferably hydrocortisone. These molecules are involved in carbohydrate, protein and fat metabolism, are anti-inflammatory and immunosuppressive, and are thought to regulate the levels of certain cytokines and/or their receptors, such as TNF-alpha, GM-CSF and G-CSF. A corticoid such as hydrocortisone can be used at a concentration of about 0.05 - 5 µM, or preferably 0.3 - 1 µM, or particularly preferably at about 0.5 µM.

The following table provides a summary list of some particularly preferred components along with suitable concentrations, preferred concentrations and the most preferred concentrations for each component. These quantities are applicable to equivalent components of each type defined herein listed in the table.

Table of Culture Components and Concentrations

Component	Suitable Exemplary Conc.	Preferred Conc.	Conc.
Standard Culture Medium (e.g., Iscove's Modified Dulbecco's Medium)	0.7 - 1.17 x	0.8 - 1.09 x	1 x
Serum albumin (e.g., bovine)	> 1 mg/ml	3 - 50 mg/ml	10 mg/ml
Transferrin (e.g., bovine)	> 25 µg/ml	25 - 1000 µg/ml	100 µg/ml
Source of lipids (e.g., soybean lipids)	> 5 µg/ml	5 - 100 µg/ml	25 µg/ml
Cholesterol	> 1 µg/ml	3 - 30 µg/ml	7 .8 µg/ml
Reducing Agent (e.g., 2-mercaptoethanol)	10 - 400 µM.	30 - 300 µM.	100 µM
Pyruvate (e.g., sodium pyruvate)	> 20 µg/ml	30 - 500 µg/ml	110 µg/ml
Glucocorticoid (e.g., Hydrocortisone)	0.05 - 5 µM.	0.3 - 1 µM.	0.5 µM
Nucleosides (e.g.,DNA/RNA derived)	1 - 100 µg/ml	5 - 30 µg/ml	10 µg/ml
Growth Factors			
(e.g., Epidermal growth factor	> 0.5 ng/ml	5 - 200 ng/ml	15 ng/ml
Fibroblast growth factor	> 0.1 ng/ml	0.5 - 40 ng/ml	2 ng/ml
Platelet-derived growth factor	> 0.5 ng/ml	2 - 200 ng/ml	10 ng/ml
Insulin)	> 0.5 µg/ml	2 - 100 µg/ml	10 µg/ml

(Table of Culture Components and Concentrations Continued)

Extracellular Matrix Materials			
(e.g., Collagen IV	> 1 µg/cm^2	2 - 100 µg/cm^2	5 µg/cm^2
Fibronectin)	> 0.2 µg/cm^2	0.5 - 100 µg/cm^2	2 µg/cm^2

One specific, exemplary cell culture medium within the scope of the present invention is set forth as follows:

Component	Concentration (mg/L) (unless stated otherwise)
ISCOVE'S MODIFIED DULBECCO'S MEDIUM	
INORGANIC SALTS:	
$CaCl_2$ (anhydr.)	165.00
KCl	330.00
KNO_3	0.076
$MgSO_4$ (anhydr.)	97.67
NaCl	4505.00
$NaHCO_3$	3024.00
NaH_2PO_4 . H_2O	125.00
Na_2SeO_3 . 5 H_2O	0.0173
OTHER COMPONENTS:	
D-Glucose	4500.00
Phenol red	15.00
HEPES	5958.00
Sodium pyruvate	110.00

AMINO ACIDS:	
L-Alanine	25.00
L-Asparagine . H_2O	28.40
L-Arginine . HCl	84.00
L-Aspartic acid	30.00
L-Cystine . 2HCl	91.24
L-Glutamic acid	75.00
L-Glutamine	584.00
Glycine	30.00
L-Histidine HCl . H_2O	42.00
L-Isoleucine	105.00
L-Leucine	105.00
L-Lysine HCl	146.00
L-Methionine	30.00
L-Phenylalanine	66.00
L-Proline	40.00
L-Serine	42.00
L-Threonine	95.00
L-Tryptophane	16.00
L-Tyrosine (Disodium salt)	104.2
L-Valine	94.00
VITAMINS:	
Biotin	0.013
Ca D-Pantothenate	4.00
Choline chloride	4.00
Folic acid	4.00
i-inositol	7.20
Nicotinamide	4.00
Pyridoxal HCl	4.00
Riboflavin	0.40
Thiamine HCl	4.00
Vitamin B_{12}	0.013

ADDITIONAL COMPONENTS

Bovine Serum Albumin	10000.00
Bovine Transferrin	100.00
Soybean Lipids	25.00
Cholesterol	7.80
Hydrocortisone	0.24
2-mercaptoethanol	7.84
Sodium Pyruvate	110.00
Adenosine	10.00
Cytidine	10.00
Guanosine	10.00
Uridine	10.00
2'-deoxyadenosine	10.00
2'-deoxyguanosine	10.00
2'-deoxycytidine	10.00
Thymidine	10.00
Epidermal growth factor	15×10^{-3}
Fibroblast growth factor	2×10^{-3}
Platelet-derived growth factor	10×10^{-3}
Insulin	10.00
Collagen IV	5 $\mu g/cm^2$
Fibronectin	2 $\mu g/cm^2$

D. Preparation of the Media and Culture Technique

The following is an exemplary method for preparing the media and carrying out the culture technique of the present invention. Equivalent components as set forth herein could be substituted within the concentration ranges set forth herein for those described below.

Extracellular matrix (ECM)

The evening before initiating the cultures, six well plates (Corning, 10 cm^2 surface area/well) are coated with 0.73 ml solution of Iscove's Modified Dulbecco's Medium (IMDM) containing glutamine (292 µg/ml), penicillin G (100 units/ml) and streptomycin sulfate (100 µg/ml), all three are supplied together (Irvine Scientific) at a 100x concentration. Dissolved extracellular matrix materials can then be added to this solution. A number of ECM molecules have been tested. It has been determined that a source of collagen is important, mouse collagen IV (5 $\mu g/cm^2$ Sigma, Cat. #C0543) being preferred. Additional ECM molecules can then be added, e.g. fibronectin and laminin, mouse fibronectin (2 $\mu g/cm^2$ Sigma, Cat. #F1141) in combination with collagen IV is used normally, in a final volume of 0.8 ml.

The next morning the wells are washed once with IMDM and the serum-free medium and cells added.

Nutrient/Metabolic Additives

A number of nutrients are added to the medium, they are prepared at a 100x final concentration, the final concentrations in the culture are shown below:

- Bovine serum albumin (10 mg/ml, Sigma, cat.# A8412).
- Bovine (100 µg/ml, Sigma cat.# T-1283) or human transferrin (300 µg/ml, Boehringer-Mannheim cat.# 652 202) (for mouse and human cultures, respectively).
- Soybean lipids (25 µg/ml, Boehringer-Mannheim cat.#652 229).